Selective Purification of Recombinant Neuroactive Peptides Using the Flagellar Type III Secretion System

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ABSTRACT The structure, assembly, and function of the bacterial flagellum involves about 60 different proteins, many of which are selectively secreted via a specific type III secretion system (T3SS) (J. Frye et al., J. Bacteriol. 188:2233–2243, 2006). The T3SS is reported to secrete proteins at rates of up to 10,000 amino acid residues per second. In this work, we showed that the flagellar T3SS of *Salmonella enterica* serovar Typhimurium could be manipulated to export recombinant nonflagellar proteins through the flagellum and into the surrounding medium. We translationally fused various neuroactive peptides and proteins from snails, spiders, snakes, sea anemone, and bacteria to the flagellar secretion substrate FlgM. We found that all tested peptides of various sizes were secreted via the bacterial flagellar T3SS. We subsequently purified the recombinant μ-conotoxin SIIIA (rSIIIA) from *Conus striatus* by affinity chromatography and confirmed that T3SS-derived rSIIIA inhibited mammalian voltage-gated sodium channel $Na_V1.2$ comparably to chemically synthesized SIIIA.

IMPORTANCE Manipulation of the flagellar secretion system bypasses the problems of inclusion body formation and cellular degradation that occur during conventional recombinant protein expression. This work serves as a proof of principle for the use of engineered bacterial cells for rapid purification of recombinant neuroactive peptides and proteins by exploiting secretion via the well-characterized flagellar type III secretion system.

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Although large strides have been made in the recombinant expression of proteins, the efficient expression of certain classes of proteins remains a challenge. These include the small, highly stable pharmacologically active polypeptides with a high density of disulfide cross links. A major group within this general class includes the polypeptides present in animal venoms. Although several different phylogenetic lineages have evolved venoms independently, all polypeptides found have convergently evolved a common set of properties that allow them to be exceptionally stable upon injection into another organism. These polypeptides are of increasing interest, because many of them have novel pharmacological activity and therefore serve as useful ligands in basic research or have direct diagnostic and therapeutic applications. Furthermore, in contrast to some neuroactive toxins, such as tetrodotoxin or saxitoxin, conopeptides do not pass the gut, thus minimizing potential toxicity. Conopeptides also have a medical advantage in that they are similar to endogenous peptide ligands (enkephalins) that lack addictive properties compared to other receptor-binding ligands that are addictive, such as heroin or morphine. One of these peptides, MVIIA, a 25-amino-acid peptide with three disulfide bonds, has become an approved drug for intractable pain (1–3).

When recombinant expression of small disulfide-rich polypeptides is attempted, the yields are generally low. A fundamental problem is that when expression levels are high, the resulting high concentrations of polypeptide in the cell lead to the formation of intermolecular aggregates, and recombinant polypeptides are found mostly in inclusion bodies. The ability to recover the polypeptide from an inclusion body in a biologically active form is not predictable and requires additional steps that vary depending on the polypeptide expressed.

In this work, we initiated a new approach that uses an expression system that should, in theory, bypass the inclusion body problem of recombinant small peptide expression. It exploits the flagellar secretion system of *Salmonella enterica* serovar Typhimurium (reviewed in references 4 and 5) that has been shown to export nonflagellar proteins if fused to flagellar secretion signals, e.g., hook protein FlgE (6) or flagellin FliC (7). The flagellar secretion system is a member of a family of bacterial type III secretion systems that selectively secrete proteins from the cytoplasm to the external medium. Almost all type III secretion systems characterized thus far are either required for the secretion of virulence determinants for a number of plant and animal pathogens or for the secretion of proteins required for the structure and function of the bacterial flagellum. About 60 genes are involved in assembly and regulation of the bacterial flagellum (8). The overall structure is composed of an external helical filament, extending many body lengths from the cell surface, attached to a rotary motor embedded

within the cell wall and membranes. For *Salmonella*, a chemosensory system controls the clockwise and counterclockwise direction of flagellar rotation, which allows the bacterium to migrate toward attractants, such as nutrients, or away from repellents that are harmful to the bacterium.

The specificity for flagellar substrate secretion is primarily determined by an N-terminal peptide secretion signal that has a disordered structure. Many secretion substrates also require a specific secretion chaperone to facilitate secretion. One flagellar secretion substrate, FlgM, is a regulatory protein that couples flagellar assembly to gene expression (9–12). σ^{28}, a transcription factor specific for flagellar promoters, directs transcription of genes specifically needed after completion of the flagellar hook-basal body structure (HBB) and thereby induces expression of the filament and chemosensory genes. FlgM binds σ^{28} and prevents its association with RNA polymerase. Completion of the flagellar hook results in a change in substrate specificity of the flagellar secretion apparatus, switching from secretion of proteins required for hook-basal body assembly to late substrate secretion of proteins needed in filament assembly. FlgM itself is a late secretion substrate and its secretion releases σ^{28}, enabling it to transcribe the filament and chemosensory genes only upon completion of the HBB (11, 13). At the same time, the σ^{28} transcription factor acts as the secretion chaperone that facilitates the secretion of its inhibitor, FlgM (14).

In this work, we utilized the flagellar FlgM protein as a vector for the secretion of the small, highly stable pharmacologically active polypeptides that contain a high density of cysteine residues, which form disulfide cross-links in the mature product. As a proof of principle, we engineered a bacterial secretion system for the recombinant expression of μ-conotoxin SIIIA in *Salmonella* Typhimurium. Using the flagellar type III secretion (T3S) apparatus, the recombinant conopeptide was selectively secreted into the culture medium (Fig. 1).

RESULTS AND DISCUSSION

Conopeptides are synthesized in the venom duct of marine cone snails and target ion channels, including voltage-gated sodium channels (VGSCs) (15). SIIIA is a μ-conotoxin from *Conus striatus* that inhibits tetrodotoxin (TTX)-resistant VGSCs in frog (16) and TTX-sensitive VGSCs in rodents (17). The μ-conotoxin SIIIA binds to site 1 of the α-subunit of VGSCs (18–20) and thereby occludes the channel pore and blocks sodium conductance. Under physiological conditions, two forms of SIIIA exist, a precursor form and a mature peptide, which has been modified by processing the C-terminal glycine-arginine residues to an amino group and changing the N-terminal glutamine residue to pyroglutamate. In this work, we recombinantly expressed two forms of SIIIA, the precursor peptide and a peptide that most closely resembles the physiological mature form (N-terminal glutamate instead of pyroglutamate). In mammalian preparations, μ-SIIIA effectively blocks neuronal sodium channels, such as $Na_V1.2$ and $Na_V1.6$, and the skeletal muscle subtype $Na_V1.4$ (17). Because SIIIA targets VGSCs and has potent analgesic activity in mice (21), it is a promising peptide for medical research and drug discovery (22). The fundamental advantage in developing our T3SS bacterial expression system is that after translation the polypeptides translocate through a narrow secretion channel in the flagellar structure and thereby bypass the problem of aggregation in inclusion bodies. Polypeptide secretion should prevent inter-

molecular aggregation in the cytoplasm. Intramolecular disulfide bonds should form as the polypeptide chain exits the reductive environment of the bacterial cytoplasm and enters an oxidizing extracellular environment.

We focused on assessing how efficiently different constructs serve as type III secretion substrates. The SIIIA pretoxin (μ-SIIIApre in Table 1 and henceforth referred to as rSIIIA) is a small 22-amino-acid peptide which was fused to FlgM with an N-terminal TEV cleavage site in front of SIIIA. In order to ensure proper secretion and to diminish the possibility of interference with the structural secretion signal of FlgM, three different constructs were designed with a polyhistidine tag at different positions. All constructs were chromosomally expressed from the arabinose-inducible promoter P_{araBAD}. Figure 1b shows the secretion profile of all three constructs. Construct 3 (FlgM-H6-TEV-SIIIA) displayed the most prominent secretion product; construct 2 (FlgM-TEV-SIIIA-H6) exhibited the lowest secretion level. This secretion ranking was consistent in all backgrounds tested, some of which carried a mutation (*fliA**, with the asterisk indicating the mutation [H14D]) in the σ^{28} structural gene *fliA* that enhances stability of this FlgM secretion chaperone protein (9). As expected, FlgM levels were very low in a strain unable to form a flagellar structure ($\Delta fliF$ strain) compared to those in a wild-type strain.

The flagellum-specific transcription factor σ^{28} acts as a chaperone to facilitate FlgM secretion. As shown in Fig. 1c, σ^{28} H14D, with its enhanced stability (10), increased levels of intracellular and secreted FlgM-SIIIA. σ^{28} H41D also increases FlgM stability (14). This is the likely cause of the increased levels of FlgM-SIIIA in the *fliA** background (Fig. 1c, lanes 2 and 3). We also tested the effect of removing the late flagellar secretion substrates (and potential secretion competitors) FliC and FliD ($\Delta fliCD$). The *fliC* and *fliD* genes are divergently transcribed. However, the *fliD* gene is in an operon with *fliT*, which encodes the FliT inhibitor of all flagellar gene expression. Thus, the $\Delta fliCD$ deletion results in removal of secretion competitors for FlgM in addition to increasing the number of flagellar basal structures (11). The net effect was increased production and secretion of both FlgM and FlgM-SIIIA from the cell (Fig. 1c, lanes 2 and 4).

As FlgM-H6-TEV-SIIIA showed the highest level of secretion, this construct was used for further purification experiments. The SIIIA fusion was expressed from an arabinose-inducible promoter to allow synchronization of the high level of expression with completion of the flagellar secretion systems. Secreted FlgM-H6-TEV-SIIIA was separated from the bacterial cell culture by centrifugation and was obtained from the supernatant under native conditions by nickel-affinity chromatography. As shown in Fig. 2a, FlgM-H6-TEV-SIIIA efficiently bound to the Ni-IDA resins and was eluted with imidazole-containing elution buffer. After concentration of the eluted fraction, rSIIIA was cleaved from the FlgM-H6-TEV secretion signal using TEV protease. Western blot analysis and Coomassie staining demonstrated that TEV cleavage was complete after 3 h at room temperature (data not shown).

As with all cysteine-rich peptides, the formation of the native disulfide bonds is a crucial process in generating biologically active SIIIA. However, the optimal buffer conditions of the TEV protease require 1 mM dithiothreitol (DTT). This redox agent provides reducing power for the TEV protease but at the same time should reduce S-S bonds in the peptide of interest. For this reason, we decided to refold rSIIIA prior to electrophysiological analysis. Refolding was carried out in a redox buffer of oxidized
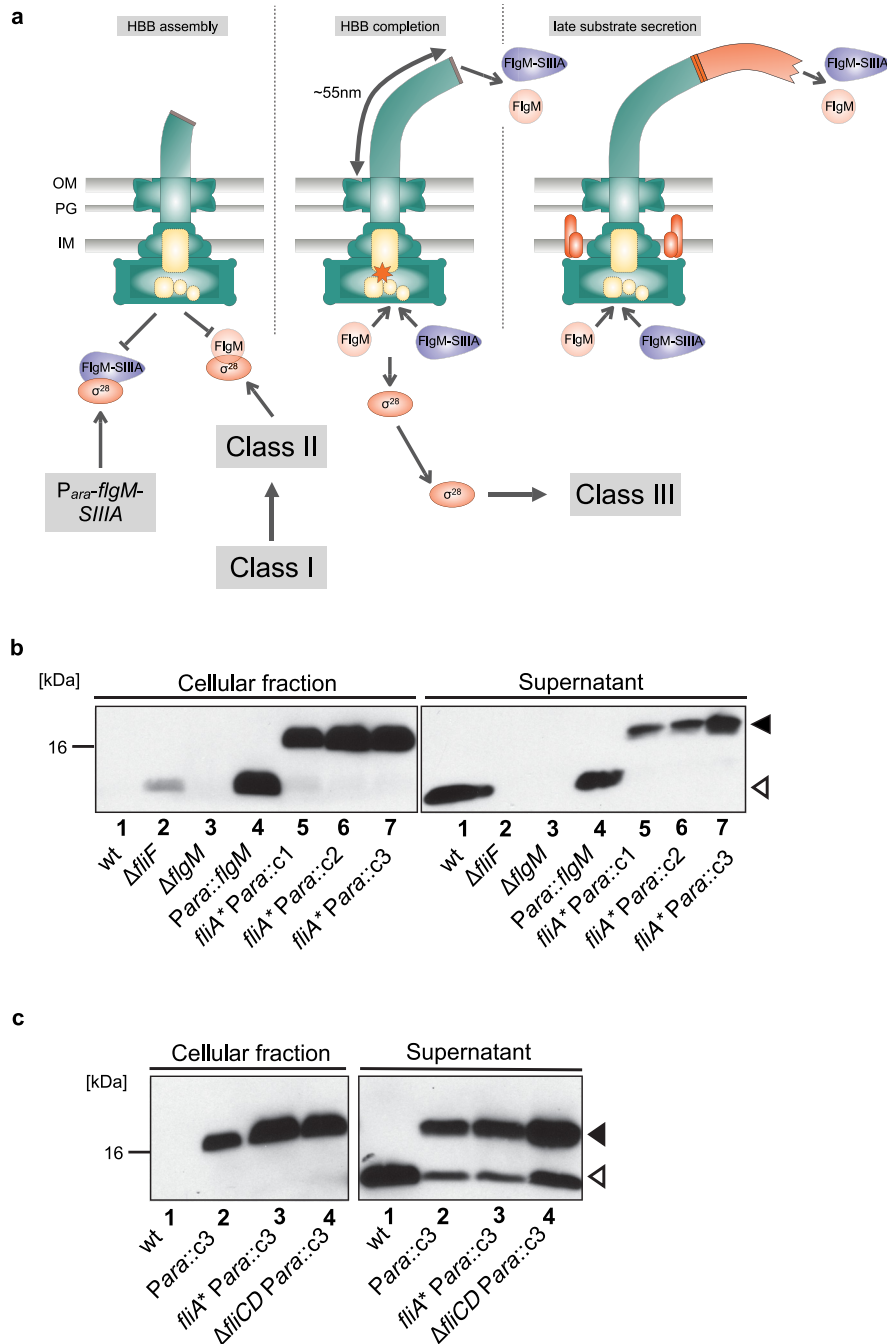
FIG 1 Engineering the flagellar type III secretion system for the secretion of SIIIA conotoxins. (a) Model: An FlgM-SIIIA translational fusion is a secretion substrate of the bacterial T3SS. The fusion construct is secreted via the flagellum-specific T3SS through the flagellar channel into the culture medium via flagellar structures that are competent for FlgM secretion. Expression of FlgM-SIIIA is induced upon addition of arabinose and is independent of flagellar class I and II gene expression. During HBB assembly, FlgM remains inside the cytosol and acts as an anti-σ^{28} factor preventing transcription of class III genes, e.g., genes encoding the flagellin subunit FliC or the stator proteins MotA and MotB. The HBB structure is completed within 30 min (39), which coincides with a substrate specificity switch within the flagellar secretion apparatus (indicated by an orange asterisk in the figure) from early to late substrate secretion. This results in secretion of FlgM and substrates needed during the final phase of flagellum assembly. OM, outer membrane; PG, peptidoglycan layer; IM, inner membrane. (b) Expression and secretion of the FlgM-SIIIA fusion protein. Secreted FlgM-SIIIA was precipitated using TCA, and immunoblots with antibodies against FlgM are shown for cellular and supernatant fractions. Protein bands of native FlgM (△) and FlgM-SIIIA (▲) fusions are marked next to the blot. Constructs 1 to 3 (labeled c1 to c3) represent the following protein fusions: c1 = H6-FlgM-TEV-SIIIA, c2 = FlgM-TEV-SIIIA-H6, c3 = FlgM-H6-TEV-SIIIA. Secretion efficiencies of three FlgM-SIIIA constructs varying in their position of the polyhistidine tag were tested. Secretion levels are shown for TH437 (wt, lane 1), TH4885 (Δ*fliF*, lane 2), TH5139 (Δ*flgM*, lane 3), TH10874 (P*ara*::*flgM*, lane 4), TH15705 (*fliA** P*ara*::c1, lane 5), TH15706 (*fliA** P*ara*::c2, lane 6), and TH15707 (*fliA** P*ara*::c3, lane 7). Wild-type FlgM bands in lanes 5 to 7 were visible upon extended exposure. (c) FlgM-H6-TEV-SIIIA was expressed from the arabinose promoter, and secretion was compared in *fliA*wt (lane 2), *fliA** (H14D, lane 3), and Δ*fliCD* (lane 4) backgrounds. Secretion of wild-type FlgM expressed from its native promoter is shown in lane 1 (TH437, labeled wt).

TABLE 1 Peptides used in this study

Peptide name	Organism	Species	Peptide size (aa)	Amino acid sequence (without modifications)
ω-MVIIA	Cone snail	*Conus magus*	25	CKGKGAKCSRLMYDCCTGSCRSGKC
ω-GVIA	Cone snail	*Conus geographus*	27	CKSPGSSCSPTSYNCCRSCNPYTKRCY
Contulakin-G	Cone snail	*Conus geographus*	16	ESEEGGSNATKKPYIL
α-Vc1.1	Cone snail	*Conus victoriae*	16	GCCSDPRCNYDHPEIC
Conantokin-G	Cone snail	*Conus geographus*	17	GEEELQENQELIREKSN
μ-SIIIAmat	Cone snail	*Conus striatus*	20	ENCCNGGCSSKWCRDHARCC
μ-SIIIApre	Cone snail	*Conus striatus*	22	QNCCNGGCSSKWCRDHARCCGR
Shk	Sea anemone	*Stichodactyla helianthus*	35	RSCIDTIPKSRCTAFQCKHSMKYRLSFCRKTCGTC
Chlorotoxin	Scorpion	*Leiurus quinquestriatus*	36	MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR
GsMTx4	Spider	*Grammostola spatulata*	35	GCLEFWWKCNPNDDKCCRPKLKCSKLFKLCNFSSG
Calciseptine	Snake	*Dendroaspis polylepis polylepis*	60	RICYIHKASLPRATKTCVENTCYKM FIRTQREYISERGCGCPTAMWPYQTECCKGDRCNK
DTA Y65A	Bacterium	*Corynebacterium diphtheriae*	190	GADDVVDSSKSFVMENFSSYHGTKPGYVDSIQK GIQKPKSGTQGNYDDDWKGFYSTDNKYDAAGASV DNENPLSGKAGGVVKVTYPGLTKVLALKVDNAE TIKKELGLSLTEPLMEQVGTEEFIKR FGDGASRVVLSLPFAEGSSSVEYINN WEQAKALSVELEINFETRGKRGQDAMYEYMAQACAGNR

and reduced glutathione, allowing thiol-disulfide exchange reactions under conditions that are known to favor formation of the native disulfide bonds (16).

Tests of the rSIIIA precursor form on *Xenopus* oocytes expressing rat Na$_V$1.2 demonstrated that its functional activity was similar to that of chemically synthesized SIIIA. Sodium currents in response to depolarization were recorded as described in Materials and Methods. Figure 2b presents the results from a representative experiment in which rSIIIA blocked Na$_V$1.2 conductance (compare the peak current of the oocyte before [gray trace] and after [black trace] rSIIIA exposure). The kinetics of the onset of block following exposure to 10 μM rSIIIA and the recovery from block following its washout were assessed as described in Materials and Methods. The observed rate constant for the onset of block (k_{obs}) and the rate constant for recovery from block (k_{off}) were, respectively, 0.17 ± 0.04 min^{-1} and 0.0043 ± 0.0004 min^{-1} (mean ± standard deviation [SD]; n = 3 oocytes). The k_{off} value is close to that observed with chemically synthesized SIIIA; however, the k_{obs} value is about 6-fold lower than that observed with chemically synthesized SIIIA (17). The difference in k_{obs} is thought to be due to the disparity in the sequences of the two peptides (N-terminal glycine carryover after TEV cleavage and lacking posttranslational modifications), and experiments are under way to test this assumption.

In addition to the secretion of SIIIA pretoxin, several other peptides from various organisms, such as spider (GsMTx4), scorpion (chlorotoxin), snake (calciseptine), snails (ω-MVIIA, ω-GVIA, contulakin-G, α-Vc1.1, conantokin-G, mature SIIIA), and sea anemone (Shk) were tested for recombinant expression and subsequent secretion in a complementary approach (see Table 1 for a detailed list). Although there were differences in cellular levels and secretion efficiencies, the results shown in Fig. 2c demonstrate that all tested peptides were secreted into the culture supernatant. We also tested the secretion of the unstable 190-amino-acid-long catalytic subunit of diphtheria toxin fused to FlgM (Fig. 2d). Notably, the proteolytic degradation that was observed within the cytosolic fraction did not occur once the protein was secreted from the cell.

For further optimization of the *Salmonella* secretion strain, a nonmotile polyhook mutant was constructed that had the following characteristics. It lacked the flagellum-related SPI-1 (Δ*prgH-hilA*) virulence system, as well as all class 3 flagellar genes (Δ*flgKL*, Δ*fljB-T*, Δ*flgMN*, Δ*aer-mcpC*, P*motA*, Δ*motA-cheZ*, Δ*mcpA*, Δ*mcpB*, Δ*tsr*) that could diminish or interfere with flagellar type III secretion of the toxin fusion of interest. In addition, the strain harbored a mutation in the *flhD* promoter (P*flhD*) and was deleted for negative regulators of the flagellar master regulator FlhD$_4$C$_2$ (Δ*lrhA*, Δ*ydiV*, Δ*ecnR*). LrhA acts as a negative regulator of *flhDC* transcription by directly binding to the promoter region of the master regulator (23). The EAL domain protein YdiV posttranslationally regulates the activity of the FlhD$_4$C$_2$ protein by targeting the complex for ClpXP-dependent degradation and thereby negatively influences FlhDC-dependent flagellar class 2 promoter activity (24, 40). Transposon insertions in those negative regulators of FlhDC have been shown to increase flagellar gene expression (25, 26).

As shown by fluorescent labeling of the flagellar hook structures in Fig. 3a, these mutations resulted in a roughly 2-fold increase of flagellar secretion structures per cell compared to wild-type cells, which in turn led to a substantial increase in the secretion rate of flagellar proteins. Secretion rates of FliK and FlgM-SIIIA (construct 3) were roughly 2-fold higher in the polyhook background than in the wild-type and *fliA** backgrounds (Fig. 3b).

The major advantage of this system is the workflow. After induction with arabinose, the bacterial cells express recombinant polypeptides and subsequently secrete the toxin into the medium. Contrary to the purification of neuropeptide by traditional methods (27), this makes it unnecessary to lyse cells in order to purify peptides. Instead, recombinant SIIIA was selectively secreted via the flagellar T3SS and it accumulated in the culture supernatant. After several hours of production, cells were removed by simple centrifugation and filtration. Other important aspects of this system are type III secretion chaperones that facilitate secretion or increase the stability of the secretion substrates. Type III secretion substrates are usually secreted in an unfolded or partially folded
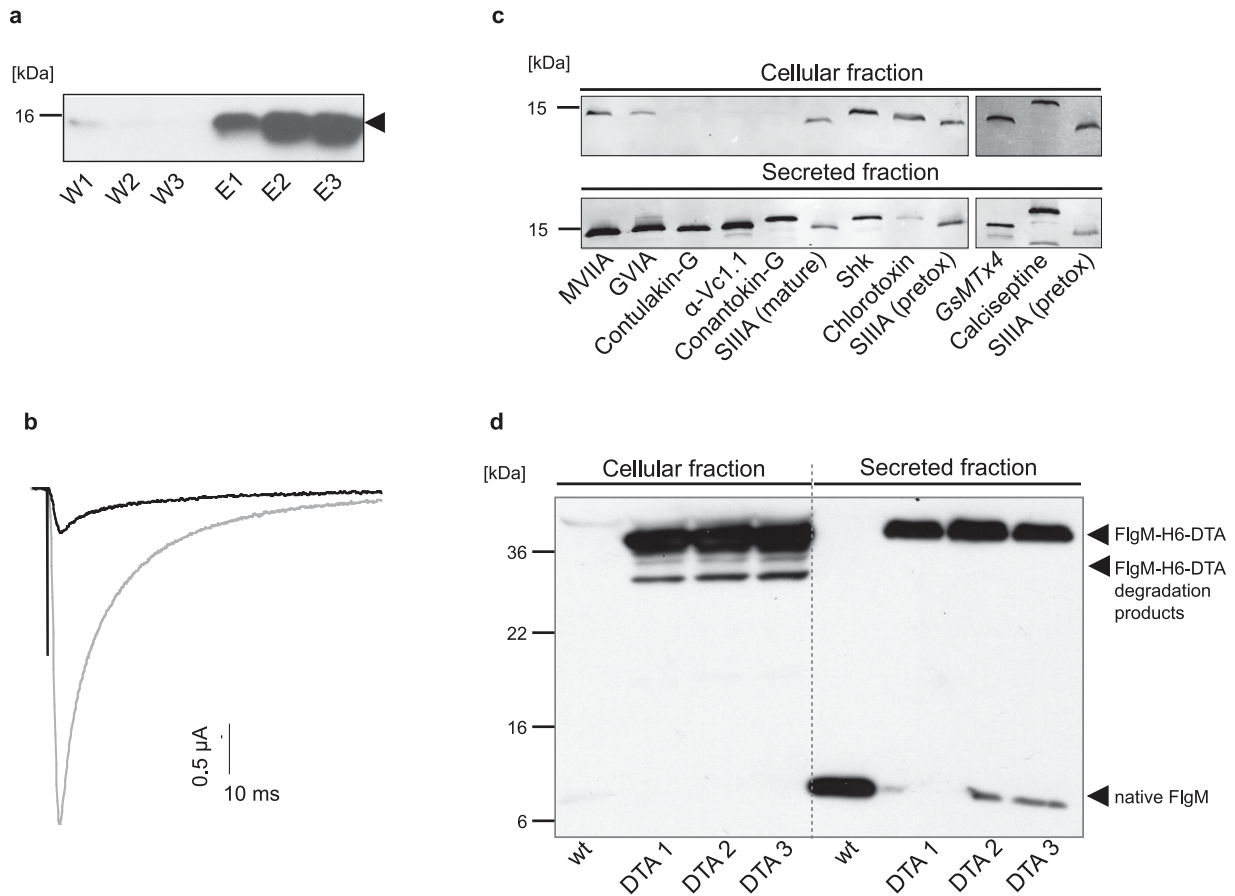
FIG 2 Purification and electrophysiology of recombinant conotoxin SIIIA and secretion of toxins from various organisms via the flagellar T3SS. (a) The supernatant of a strain expressing and secreting recombinant SIIIA fused to FlgM (TH15707 *fliA** [H14D] Δ*araBAD1036::flgM-H6-TEV-SIIIA*) was filtered and bound to an Ni^{2+}-IDA column as described in Materials and Methods. The matrix was washed after binding (W1 to W3), and FlgM-SIIIA was eluted in three steps with imidazole-containing elution buffer (E1 to E3). For Western blot detection, samples were TCA precipitated. Due to the increased FlgM-SIIIA concentration in the elution fractions, only 1/10 of the volume was used for TCA precipitation of elution fractions 1 to 3. (b) Recombinant SIIIA blocks voltage-gated sodium channel $Na_V1.2$. A *Xenopus* oocyte expressing rat $Na_V1.2$ was exposed to 10 μM rSIIIA, while sodium currents were monitored as described in Materials and Methods. Currents recorded before toxin exposure (control, gray trace) and following ~20-min exposure to 10 μM rSIIIA (black trace). Each trace represents the average of five responses. The difference of the peak values between the two traces corresponds to the inhibitory effect rSIIIA has on channel $Na_V1.2$. FlgM-H6-TEV was translationally fused to six different peptides from cone snails and one peptide each from a sea anemone, scorpion, spider, and snake (c), and FlgM-H6 was fused to *Corynebacterium diphtheriae* (d) (see also Table 1 for a detailed list). Peptides were expressed in a *Salmonella* polyhook background (see text for details). Secretion of diphtheria toxin fragment A from *Salmonella* strain TH16229 was tested using three independent biological replicates (labeled 1 to 3). Secretion of recombinant peptides was performed as described before.

state, which is often achieved by interaction of the chaperones with the secondary structure of the secreted protein. Since SIIIA was fused to FlgM, a type III secretion substrate, it was likely protected from premature folding by interaction with FliA (σ^{28}), the cognate type III secretion chaperone of FlgM (14). In the past, shorter fragments of FliC containing only the presumed type III secretion signal were used as a secretion shuttle (7). For our recombinant expression, we intentionally used full-length FlgM, which increased secretion efficiency due to the presence of its FliA chaperone-binding site.

In summary, this system provides an easy-to-use method for complicated peptide purification—a task that goes beyond yield and solubility of peptides and requires the peptides and proteins to be accessible in a correctly folded and active state. As shown here, this technique could be of use for the diverse array of different bioactive small peptides that is especially apparent within the venom of cone snails. More than 500 species

of fish-, mollusk-, and worm-hunting cone snails have evolved, and every *Conus* species appears to have a repertoire of around 100 different venom components, mostly small, disulfide-rich peptides that rapidly affect the prey organism at multiple targets. Each neuroactive peptide of the venom is highly specific for an individual channel isoform, and it is estimated that >50,000 conopeptides exist, but less than 0.1% have been characterized pharmacologically (22, 28). To make use of this potential, it is necessary to isolate and characterize the target and mode of action of single venom components. The identification of neuroactive ligands has a potential to make a significant contribution to understanding the molecular basis of electrical signaling in the nervous system of mammals. Additionally, individual peptides come into consideration as potential pharmaceutical agents—as analgesics and as drugs for the treatment of various diseases such as epilepsy, autoimmune diseases, or even spinal cord injuries.

a

Membrane
(FM-64)

DNA
(Hoechst)

Flagellar Hooks
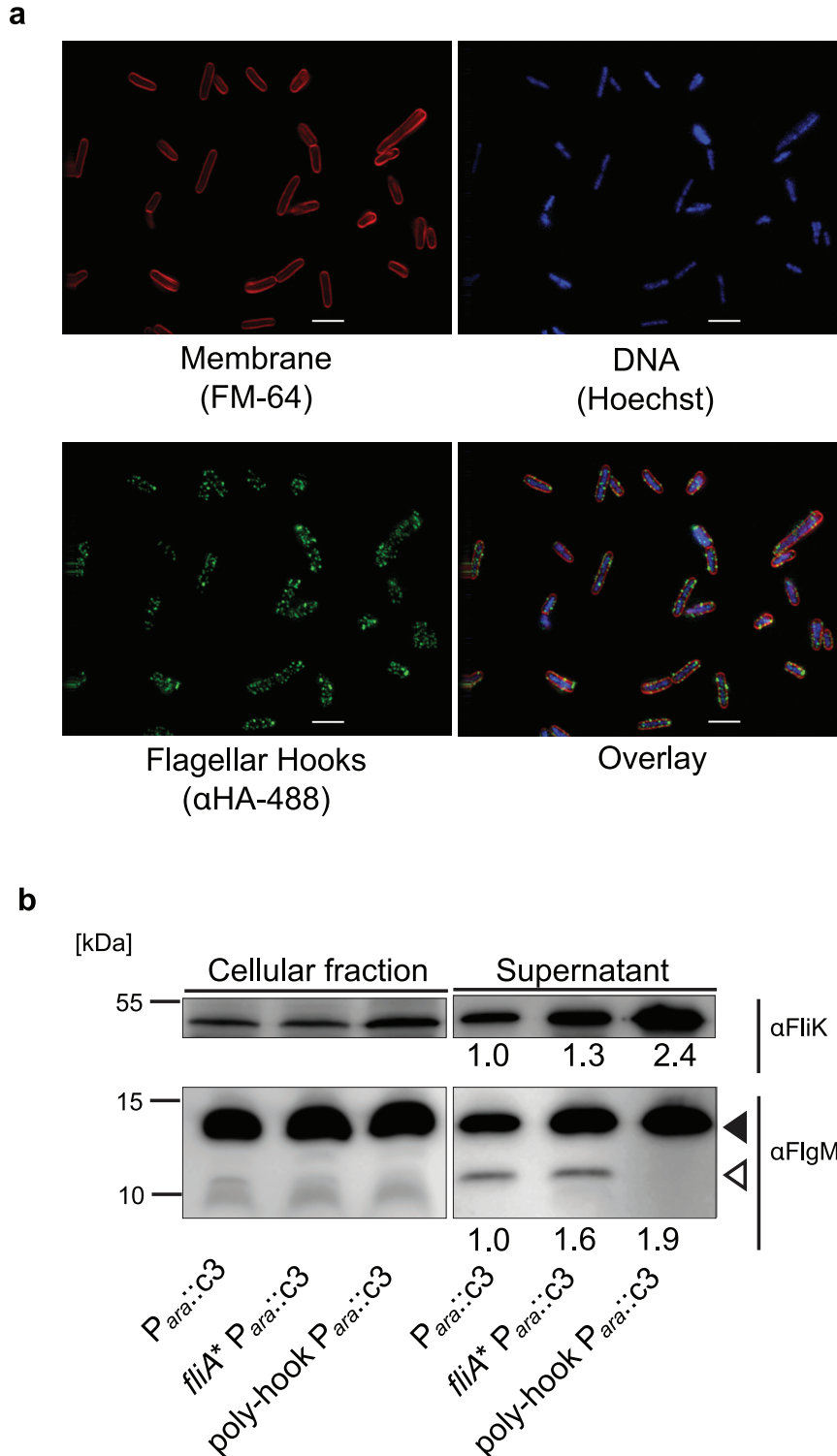(αHA-488)

Overlay

b

FIG 3 Effect of *Salmonella* polyhook background on secretion. (a) Immunostaining of the polyhook background TH16778 used for secretion of FlgM-H6-TEV-toxin fusions. A three-fold repeat of a hemagglutinin epitope tag was inserted into the flagellar hook subunit to facilitate hook detection by fluorescent microscopy. Red, membrane stained with FM-64; blue, DNA stained with Hoechst; green, flagellar hook-basal-body complexes (*flgE*::3×HA) labeled with antihemagglutinin antibodies coupled to Alexa Fluor 488. The scale bar is 5 μm. (b) Secreted proteins were precipitated using TCA, and immunoblots with antibodies against FlgM and FliK are shown for cellular and supernatant fractions. Protein bands of native FlgM ($\triangle$) and FlgM-SIIIA fusions ($\blacktriangle$) are marked next to the blot. Secretion levels were compared by measuring the densiometric intensity of the detected bands using ImageJ (37). Secretion values relative to the secretion in a P$_{ara}$ wild-type background (first lane) are displayed below the corresponding band.

MATERIALS AND METHODS

Bacterial strains and media. All bacterial strains used in this study are listed in Table S1 in the supplemental material. The commonly used non-pathogenic strain of *Salmonella enterica* serovar Typhimurium LT2 was used for all experiments. LT2 harbors mutations in the *ropS* and *mviA* genes that render it avirulent (29–31). The *mviA* gene encodes for the regulator of a two-component system involved in *Salmonella* virulence. The *rpoS* gene product (stationary-phase sigma transcription factor) is required for the expression of multiple virulence factors. The two mutations within the virulence pathway of *Salmonella* result in attenuation of virulence. To further ensure nonpathogenicity of the used bacterial expression strains, most experiments were performed in a strain deleted for major structural parts and the master regulator of the SPI-1 injectisome virulence system (Δ*prgH-hilA*).

Cells were cultured in Luria-Bertani (LB) medium and, when necessary, supplemented with ampicillin (100 μg/milliliter) or tetracycline (15 μg/milliliter). The generalized transducing phage of *S.* Typhimurium P22 *HT105/1 int-201* was used in all transductional crosses (32).

All work regarding *Salmonella* Typhimurium was performed with institutional approval, and in particular work with purified conotoxin SIIIA was performed under select agent rules at the University of Utah.

Construction of chromosomally expressed *flgM*-toxin fusions. SIIIA was amplified with a *de novo* fill-in PCR reaction using a long primer, SIIIA_long_fw (CAGAACTGCTGCAACGGCGGCTGCAGCAG CAAATGGTGCCGCGATCATGCGCGCTGCTGCGGCCGC), covering all 66 bp encoding SIIIA (QNCCNGGCSSKWCRDHARCCGR). The SIIIA sequence was designed according to the optimal codon usage of *Salmonella* Typhimurium. The 66-bp sequence was duplexed with the help of a short reverse primer (SIIIA_short_rv: GCGGCCGCAGCAGCG CGCATG) initiating the fill-in from the 3′ end of the template primer.

A cleavage site for the TEV protease (ENLYFQG) and a polyhistidine tag [H6 encoded by (CATCAC)$_3$, indicating a triple repeat of two codons encoding the same 6 amino acids] were inserted during amplification of *flgM* and SIIIA at various positions, resulting in three different construct (named constructs 1 to 3).

Below, the construction procedure of construct 1 will be explained exemplarily in more detail, and construct 2 and construct 3 were designed accordingly (see Table S2 in the supplemental material for primer sequences). The *flgM* gene was amplified from genomic DNA (TH2788) using forward primer 1_HS1_flgM_fw and reverse primer 1_HS2_flgM_rv. The reverse primer encoded an 18-bp overhang that was homologous to the 5′ SIIIA sequence (see above). To increase the length of the homologous region, SIIIA was amplified from the previous synthesized template with primers 1_HS3_SIIIA_fw and 1_HS4_SIIIA_rv, adding an additional 10-bp overlap with homology to the sequence of the TEV protease cleavage site. The PCR products of FlgM and SIIIA were purified and used in a subsequent fusion PCR as a template together with forward primer 1_HS1_flgM_fw and reverse primer 1_HS4_SIIIA_rv. This method allows the fusion of two PCR products that share a homology of (in case of construct 1) 28 bp, resulting in one long *flgM*-SIIIA fusion construct. All constructs contained a 5′-BamHI and a 3′-EcoRI restriction site for cloning into pUC18, resulting in the subcloning vectors (pHS1 [pUC18 BamHI-*His6-flgM-TEV-SIIIA-EcoR*I], pHS2 [pUC18 BamHI-*flgM-TEV-SIIIA-His6-EcoR*I], and pHS3 [pUC18 BamHI-*flgM-His6-TEV-SIIIA-EcoR*I]).

All *flgM*-SIIIA fusions were amplified from the respective subcloning vectors with primers having homologous regions for the native *flgM* locus or the arabinose locus (Δ*araBAD*). Chromosomal insertions were constructed using λ-Red-mediated recombination as described before (33).

The expression of SIIIA in *Salmonella* resulted in a recombinant peptide that was lacking posttranslational modifications that are usually present under physiological conditions. Those include a neutral N-terminal pyroglutamate, C-terminal amidation, and cleavage of a two-residue fragment named SIIIApre. However, according to functional studies on the related μ-conotoxin GIIIA, the potency of SIIIA is thought to be deter-mined by the lysine at position 11 (16, 34–36) and therefore should not be affected by any N- and C-terminal sequence variations.

All other peptides from various organisms (snails, spiders, snakes, and sea anemone) were constructed correspondingly, and their sequences are listed in Table 1 and Table S2 in the supplemental material.

Fragment A of the diphtheria toxin (DTA Y65A) was amplified from plasmid pTH794 with primers DTA-flgM_fw (ACTCGCTCATTCGCGA GGCGCAGAGCTACTTACAGAGTAAAGGCAGCTCTCACCACC ACC) and DTA-flgM_rv (TTCATCAACGCGCCCCCCATGGGACGCG TTTTTAGA GGCATTAACGGTTACCTGCACAAG). DTA amplification included a polyhistidine tag (H6) with a GSS linker before the His tag and an SSGLVPR linker between the His tag and the first amino acid of DTA. DTA was chromosomally inserted by λ-Red-mediated recombinat ion. The insertion was carried out in a Δ*araBAD::flgM⁺* background. DTA was targeted in front of the *flgM* stop codon, resulting in a translat ional *flgM-DTA* fusion.

Recombinant expression and purification of SIIIA conotoxin. Strains expressing SIIIA conotoxin fusions were picked from a fresh single colony and grown in 10 ml LB overnight. The overnight cultures were diluted 1:100 into 1 liter of fresh medium and grown for 6 h. If appropri ate, SIIIA expression was induced after the first 2 h by the addition of 0.2% arabinose. Cells were pelleted by centrifugation (7,000 rpm), and the supernatant containing FlgM-SIIIA was passed through a 0.22-μm poly ethersulfone filter (Corning, NY), a low-protein-binding membrane for removal of residual bacteria. For further purification, a gravity flow column (Bio-Rad) packed with 3 g Ni-IDA resin (Protino Ni-IDA; Machery-Nagel) was used, and affinity-tagged proteins were eluted under native conditions at pH 7.5 with a buffer containing 250 mM imidazole.

Secretion assay. Overnight cultures were diluted 1:100 in LB and grown for 2 h at 37°C before inducing the expression of the respective FlgM-toxin fusion by adding 0.2% l-arabinose. Cells were kept at 37°C for an additional 4 h, while the fusion proteins were expressed. After a total of 6 h, the optical density at 600 nm (OD_{600}) was determined for all strains.

Two-milliliter aliquots of the resulting cell culture were centrifuged for 10 min at 4°C and 7,000 rpm to obtain, for each aliquot, a pellet and supernatant. The supernatant was filtered through a low-protein-binding filter with a 0.2-μm pore size (Acrodisk syringe filter; Pall Life Sciences) to remove the remaining cells. Alternatively, aliquots were centrifuged twice at maximum speed to remove residual cells. Secreted proteins in the fil-tered or twice-centrifuged supernatant were precipitated by the addition of TCA (10% final concentration). The supernatant samples were resus-pended in 2× SDS sample buffer (100 mM Tris [pH 6.8], 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 25 mM EDTA, 0.04% bromophenol blue) and adjusted to 20 OD_{600} units/μl. The cellular pellet fraction was suspended in 2× SDS sample buffer, whose volume was adjusted to yield 20 OD_{600} units/μl.

SDS-PAGE and Western blotting. Expressed FlgM-toxin fusions of whole-cell lysate and cultural supernatant were subjected to SDS poly-acrylamide gel electrophoresis and analyzed by immunoblotting using polyclonal anti-FlgM and polyclonal anti-FliK antibodies (rabbit) for de-tection. Antigen-antibody complexes were visualized by chemilumines-cent or infrared detection using the LI-COR Odyssey imaging system. For chemiluminescent development, secondary goat α-rabbit antibodies (Bio-Rad) conjugated with horseradish peroxidase (HRP) and an ECL detection kit (Amersham Biosciences) were used. For infrared detection, secondary anti-rabbit IRDye690 (LI-COR) was used. Densiometric mea-surements of FlgM-SIIIA and FliK bands were performed using ImageJ 1.45s for Mac OS X (37).

TEV protease cleavage. SIIIA was cleaved off flagellar secretion sub-strates using AcTEV protease (Invitrogen). A total of 450 unified atomic mass units of TEV protease was added to 15 ml of the elution fraction, which corresponds to the secreted proteins from a 750-ml culture. Cleav-age was performed overnight at room temperature in elution buffer con-taining 1 mM DTT and 0.5 mM EDTA.

Folding of SIIIA. Recombinant SIIIA was folded in 0.1 M Tris buffer, pH 7.5, using 1 mM oxidized glutathione (GSSG) and 0.1 mM EDTA. The folding reaction was allowed to proceed overnight at room temperature and was quenched by the addition of formic acid to a final concentration of 8%.

Solid-phase extraction of SIIIA. C_{18} solid-phase extraction columns (Supelclean LC-18; Supelco) were used to remove the oxidized peptide from the other components of the folding and cleavage reactions. The isolated peptide was then dried and purified by high-performance liquid chromatography (HPLC) on a Waters 600 chromatograph, equipped with a dual-wavelength absorbance detector and a Vydac C_{18} analytical column. The HPLC separation was run with a linear gradient from 4.5% to 31.5% aqueous (aq) acetonitrile (0.9% change per minute), maintaining 0.1% trifluoroacetic acid (TFA) throughout. This allowed purification of the correct folding product; mass was validated by matrix-assisted laser desorption ionization--time of flight mass spectrometry (MALDI-TOF).

Electrophysiology of mammalian Na_V channels. The functional activity of recombinant SIIIA was assessed electrophysiologically with *Xenopus* oocytes expressing $Na_V1.2$, as described before (38). Briefly, the oocyte was placed in a 30-μl recording chamber perfused with ND96 and two-electrode voltage clamped. A holding potential of -80 mV was used, and activation of sodium channels was achieved by stepping the potential to -10 mV for 50 ms every 20 s. Exposure to toxin was performed in a static bath to conserve peptide, as follows. Prior to toxin application, the perfusion of the bath was halted, and control responses were recorded. Peptide (3 μl at 10 times the final concentration) was then applied to the bath, which was stirred for 5 s using a micropipette. Recordings of the onset of block by the peptide were obtained for about 20 min, after which the perfusion with ND96 was resumed to remove unbound peptide while the rate of recovery from block was monitored for about 20 min. The time course of the onset of block was fit to a single-exponential function to obtain the observed rate constant of block, k_{obs}. The rate of recovery from block during peptide washout was too slow to measure by curve fitting, so k_{off} was estimated from the level of recovery after 20 min of washing and assuming exponential decay of block (17). All recordings were obtained at room temperature.

Immunostaining. Fluorescent microscopy analysis was performed as described previously by Erhardt and Hughes (25).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00115-12/-/DCSupplemental.

TABLE S1, DOC file, 0.1 MB.
TABLE S2, DOC file, 0.1 MB.

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